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Changes in cell migration of mesenchymal cells during osteogenic differentiation

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ABSTRACT

We showed that the migration, morphology and adhesiveness of undifferentiated mesenchymal cells dramatically changed during osteogenic differentiation. The migration of these cells was transiently upregulated early in osteogenic differentiation. At a later stage, migration was decreased but adhesiveness was increased. Furthermore, Cdc42 and Rac1 Rho-family small GTPases were activated at early stages of differentiation and the phosphorylation level of FAK decreased as differentiation progressed. We also showed cell migration was promoted by inhibition of the Rho-ROCK-myosin signaling. Finally, using a mouse model of ectopic bone formation, we confirmed that treatment with ROCK inhibitor, Y-27632 increased cell movement into bone formation sites, resulting in enhanced osteogenesis. These results provide a new insight into the link between cell migration and osteogenic differentiation.

1. Introduction

Migration of osteoblast precursor cells is critical for bone formation and fracture healing [1,2]. In order to form the appropriate shape of the future bone, both the recruitment and the osteogenic differentiation of the precursors must be precisely regulated. Cells originating from the periosteum [3], bone marrow [4,5], circulating blood [6], and the surrounding soft tissues [7] have been reported to function as osteoblast precursors. Although a number of individual studies concerning cell migration and osteogenic differentiation have been conducted, the precise link between cell migration and differentiation has not yet been reported.

A number of factors associated with bone have been reported to induce directional migration of osteoblasts, including transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), insulin-like growth factor (IGF), bone morphogenetic protein (BMP), and vascular endothelial growth factor (VEGF) [8–12]. We have also reported chemotaxis of osteoblasts to IGF-1 that is secreted from the osteoblasts themselves [13]. We thus hypothesized that these soluble factors regulated both prior cell migration and osteogenic differentiation.

We also reported that signaling of the small GTPase Rho and its target ROCK regulates osteogenic differentiation [14]. In that study, we noticed that inhibition of Rho-ROCK signaling using Y-27632 enhanced bone mass in an ectopic bone formation model through induction of osteogenic differentiation. However, the effect of the ROCK inhibitor on precursor cell migration is unclear. Several studies have shown that ROCK inhibition reduces cell contractility, actin cytoskeleton assembly and focal adhesion turnover, all of which result in loss of cell motility [15–17]. In contrast, ROCK inhibition promotes the migration of primary rat embryo fibroblasts and osteosarcoma cells [18–21]. Thus, the effect of ROCK appears to differ depending on the cell type and the testing system used [22,23].

In this study, we determined if cell migration was altered during osteogenic differentiation using mesenchymal cell lines (ST2) and primary bone marrow derived cells (BMDCs). We also examined the effect of ROCK inhibition on the migration abilities of these cells. Our findings may lead to a new therapeutic strategy for bone formation and fracture healing.

2. Materials and methods

2.1. Osteogenic induction

ST2 cells (7.5×10^4 cells/dish) were plated in 6-cm dishes (BD Falcon, Bedford, MA). To induce osteogenic differentiation, the culture medium was changed from maintenance medium (RPMI1640

Abbreviations: ROCK, Rho-associated kinase; FAK, focal adhesion kinase; OPN, osteopontin; PTHR, parathyroid hormone receptor; Osx, osterix; OCN, osteocalcin; MLC, myosin light chain

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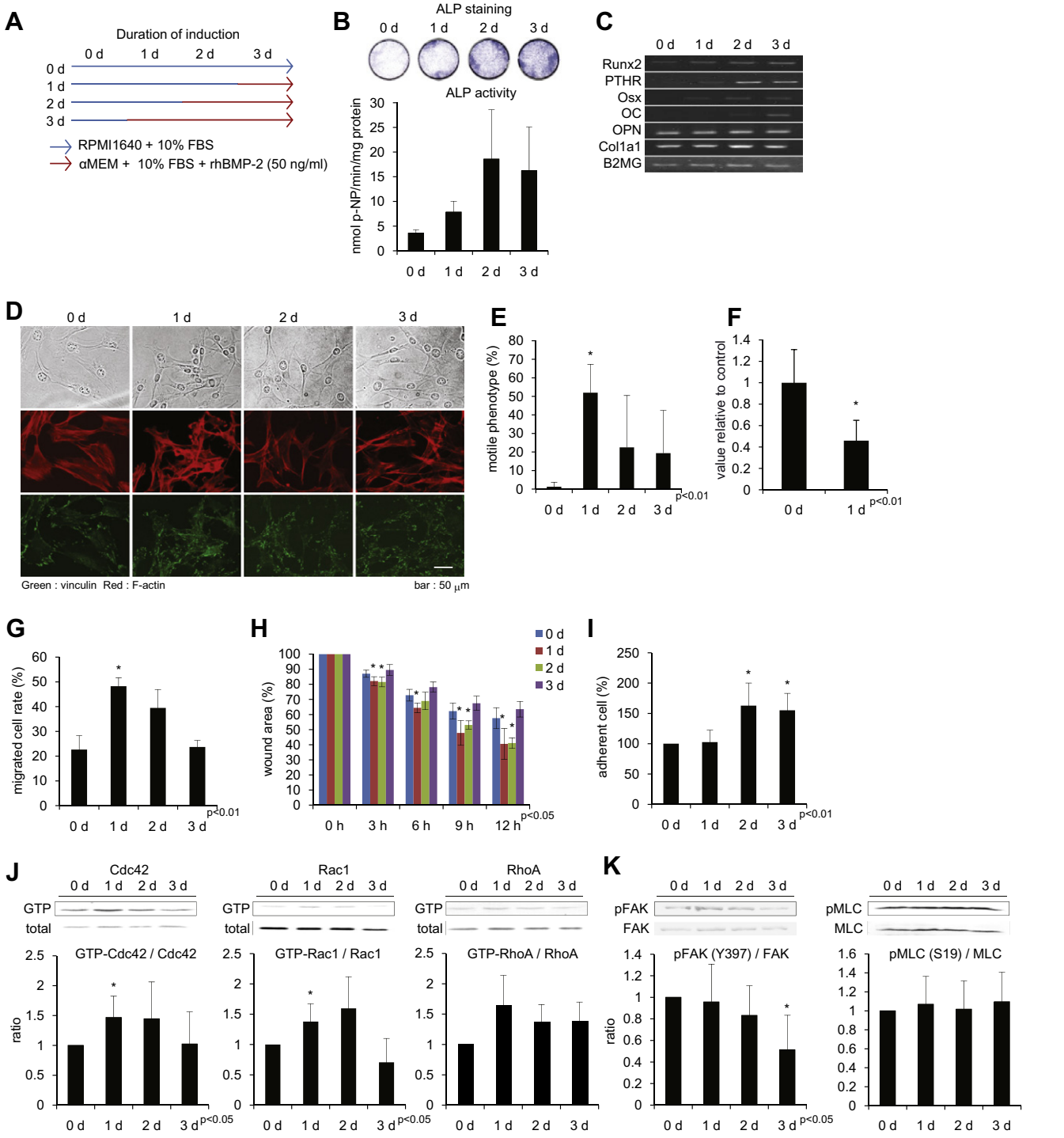

Fig. 1. Osteogenic differentiation affected cell migration ability. (A) Schematic presentation of the experimental protocol of the osteogenic differentiation assay. Osteogenic differentiation of ST2 cells was induced by treatment with rhBMP-2 (50 ng/ml). The arrows indicate the composition of the culture medium; non-inducing medium, blue; osteogenic induction medium, red. (B) ALP staining and activity during osteogenic induction over the indicated period (0–3 days) ($n = 7$). (C) RT-PCR analysis of the mRNA expression of various osteogenic marker genes. (D) Immunofluorescent staining of the actin cytoskeleton during osteogenic differentiation. Phase contrast images are shown at the top. (E) Quantification of cells with a motile phenotype ($n = 5$). (F) Quantification of cell area after osteogenic induction ($n > 100$). (G) Cell motility during osteogenic differentiation assayed using a modified Boyden chamber assay ($n = 6$). (H) Wound healing assay of differentiated ST2 cells ($n = 5$). (I) Assay of cell adhesion during osteogenic differentiation. (J) Activity of cellular Cdc42, Rac1 and RhoA was analyzed using a pull-down assay of the GTP-bound active forms. Levels of the GTP-bound (GTP) proteins and total cellular GTPases, were analyzed by Western blotting (top). (K) Western blotting analysis of the phosphorylation level of FAK and MLC, and the ratio of phosphorylated to non-phosphorylated protein during osteogenic differentiation ($n = 4$).

medium supplemented with 10% FBS), to induction medium (alpha minimal essential medium (α-MEM) (Invitrogen) supplemented with 10% FBS and 50 ng/ml rhBMP-2), and the cells were then cultured for the indicated period (0–3 days).

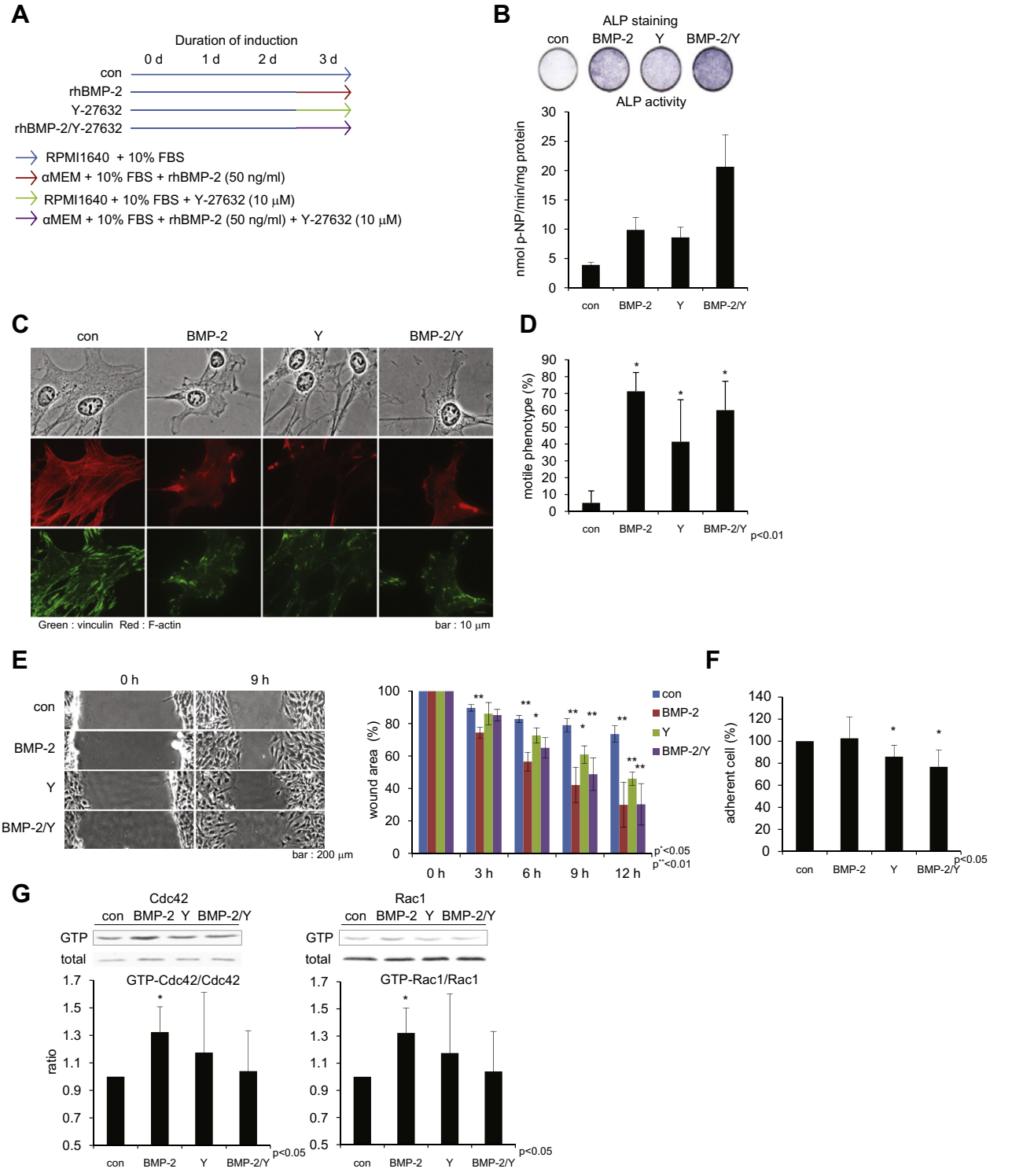

Fig. 2. The ROCK inhibitor, Y-27632, stimulated osteogenic differentiation and cell migration. (A) Schematic outline of the experimental protocol used to examine the effects of Y-27632 with or without rhBMP-2 on osteogenic differentiation and cell migration. (B) ALP staining and activity after treatment of ST2 cells with rhBMP-2 (BMP-2, 50 ng/ml) and/or Y-27632 (Y, 10 μM) ($n = 5$). Con, control. (C) Immunofluorescent staining of the actin cytoskeleton and vinculin (a marker of focal adhesions) after the indicated treatments. (D) Quantification of cells with a motile phenotype ($n = 6$). (E) Wound healing migration assay of treated cells (see also Supplemental movies 1–4) ($n = 4$). (F) Adhesion assay of treated cells. (G) Activity of Cdc42 and Rac1 detected using a pull-down assay of the GTP-bound active forms.

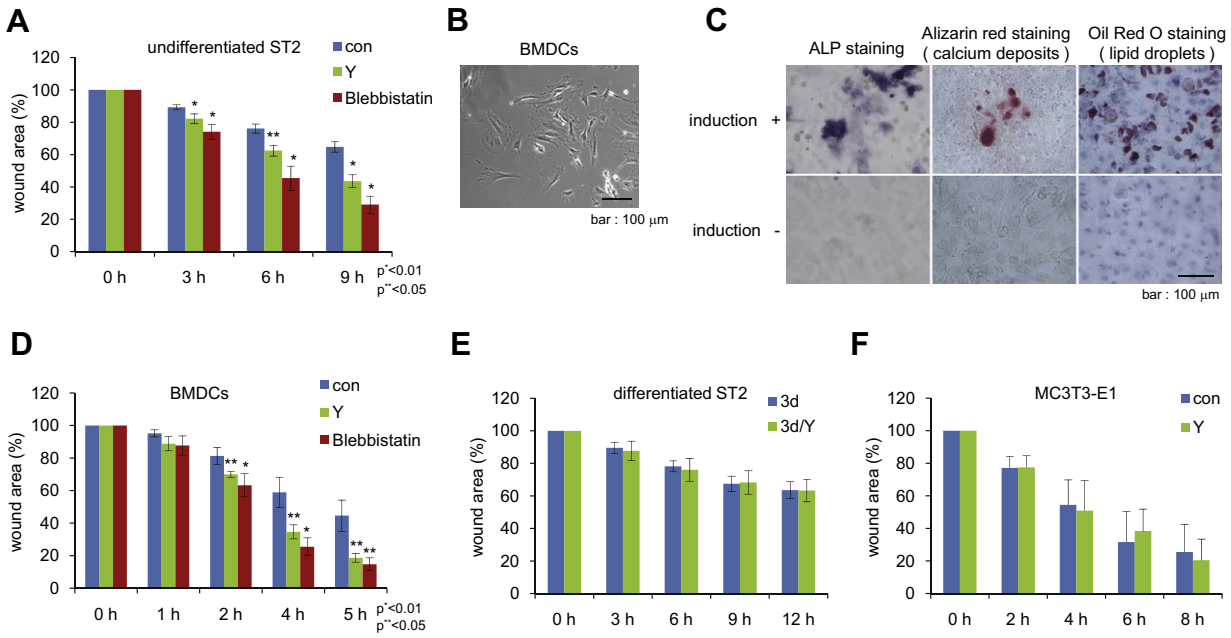

Fig. 3. Y-27632 and the myosin II inhibitor, blebbistatin, enhanced undifferentiated mesenchymal cell motility. (A) Wound healing migration assay of control ST2 cells (con) or ST2 cells treated with Y-27632 (Y) or blebbistatin ($n = 5$). (B) Phase contrast microscopy of passage-4 BMDCs prepared from C57BL/6 mice. (C) Osteogenic differentiation of BMDCs following treatment with rhBMP-2 (50 ng/ml) was analyzed by staining for ALP (cultured for 3 days) and by alizarin red S staining of calcium deposits (cultured for 15 days). Adipogenic differentiation, induced by treatment with troglitazone (2 μM) for 10 days, was assayed by staining of lipid droplets with Oil Red O. (D) Wound healing migration assay of control BMDCs (con) or BMDCs treated with Y-27632 (Y) or blebbistatin ($n = 4$). Treatment with Y-27632 (10 μM) did not enhanced cell motility of (E) differentiated ST2 cells (on days 3) ($n = 6$) or (F) MC3T3-E1 cells ($n = 8$) in a wound healing assay.

Additional materials and methods are listed in Supplementary data SD1–3.

3. Results and discussion

3.1. The motility of progenitor cells dramatically changed during differentiation

In order to investigate the link between cell migration and osteogenic differentiation, we compared the migration ability of four different mesenchymal cell lines with Boyden chamber assay using FBS as a chemoattractant. These cells were different stages of differentiation as showing several lineage specific marker expressions (Supplementary data, Fig. S1A and B). Migration abilities were varied and undifferentiated cells showed faster migration ability (Supplementary data, Fig. S1C–F). ST2 cells could differentiate into osteogenic, chondrogenic and adiogenic cells under appropriate culture conditions (Supplementary data, Fig. S1G), thus ST2 cells showed the ability of pluripotent undifferentiated mesenchymal cell. Next, we induced osteogenic differentiation of ST2 cells using rhBMP-2 is shown in Fig. 1A. Osteogenic differentiation was detected by ALP staining and measurement of ALP enzyme activity (Fig. 1B). ALP activity was increased after induction and reached a plateau on day 2 of induction. The expression of various osteogenic marker genes during differentiation was analyzed using RT-PCR (Fig. 1C). Some genes, such as Runx2, OPN, and Col1a1 were continuously expressed throughout the entire experiment. Expression of PTHR and Osx genes was detected on day 1 after induction and that of the OCN gene on day 2 after induction. The expression of all three of these genes increased along with the induction time. Thus, osteogenic differentiation was gradually induced after rhBMP-2 treatment.

We next analyzed morphological changes in these cells during differentiation. Using immunofluorescent staining, we analyzed the actin cytoskeleton and focal adhesion formation (Fig. 1D). Undifferentiated (0 d) cells showed marked actin stress fibers (red) and numerous focal adhesions (green). These structures were dramatically attenuated on day 1 of induction, which is indicative of cells with a motile phenotype, but actin stress fibers and focal adhesion formation reappeared on days 2 and 3 after induction. These changes in the motile phenotype of the cells were confirmed by quantitative analysis (Fig. 1E), and measuring cell area (Fig. 1F). We then analyzed the migration ability of these cells during induction of osteogenic differentiation using modified Boyden chamber and wound healing assays (Fig. 1G and H). Cell migration was significantly enhanced on day 1 of induction in both assays, but was decreased to the basal level by day 3 of induction. Conversely, cell adhesiveness did not change on day 1, but was upregulated on days 2 and 3 after induction (Fig. 1I). To distinguish whether the changes of mesenchymal cell migration was caused by rhBMP-2 or by the osteogenic differentiation, we checked the direct effect of rhBMP-2 on cell migration during short period (Supplementary data, Fig. S2). rhBMP-2 did not affect cell migration as a chemoattractant. Thus, the cell migration change was caused by osteogenic differentiation. Lastly, biochemical changes related to cell migration were examined in these cells. The activity of Cdc42 and Rac1 was significantly increased on day 1 of induction, similar to cell migration (Fig. 1G, H, and J). Then, it was declined the control level on day 3. By contrast, the change of the activity of RhoA was marginal during induction. The phosphorylation level of FAK, which is responsible for focal adhesion turnover, decreased as differentiation proceeded (Fig. 1K). This down-regulation of the phosphorylation level of FAK and the resultant decrease in focal adhesion turnover may account for the enhanced adhesiveness of the cells at later stages of induction (Fig. 1I). The phosphorylation level of MLC, related to actomyosin contractility, showed no significant change during induction. In addition, using video recording of the cells, we noticed that cell protrusions of individual cells were markedly enhanced on day 1 of induction (Supplementary movies 3 and 4). Based on these results, we speculated that differentiated

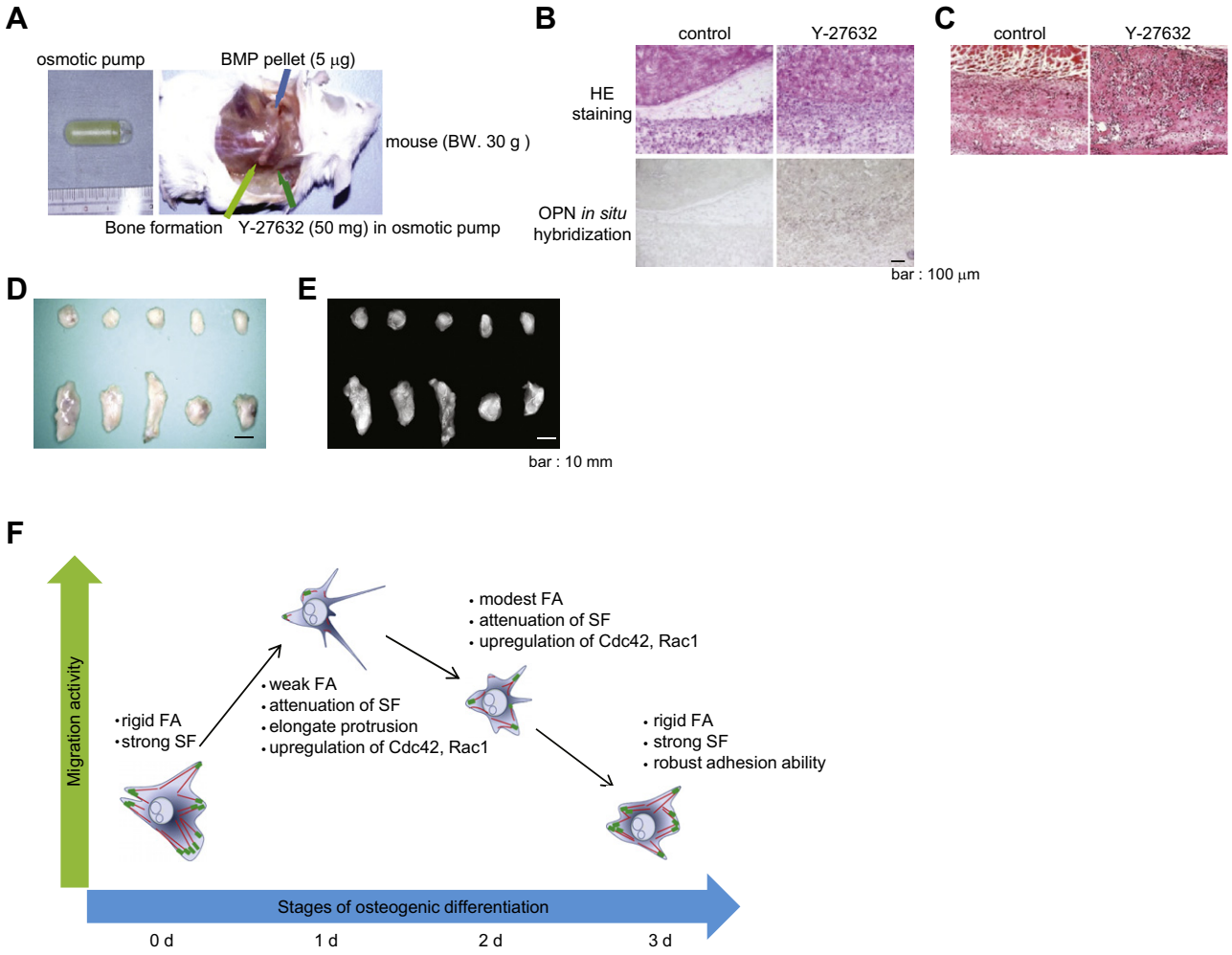

Fig. 4. Y-27632 promoted mesenchymal cell movement into a bone formation site and accelerated bone formation in mice. (A) A rhBMP-2 (5 μg)-collagen1 composite was implanted into a dorsal subfascial pocket of a mouse (body weight (BW), 30 g) and Y-27632 (50 mg) was supplied to the mouse using an osmotic pump. (B) Osteopontin-positive cells in the rhBMP-2-collagen1 composite were analyzed 4 days after implantation using Hematoxylin and Eosin (HE) staining of histological sections and osteopontin (OPN) in situ hybridization. (C) HE staining of histological sections of the BMP composite of control (left) and Y-27632-treated (right) mice at 1 week after implantation. (D) Macroscopic photograph of ectopic ossicles 1 week after implantation in control (upper) and Y-27632-treated mice (lower). (E) Radiographic image of the ossicles in control (upper) and Y-27632-treated (lower) mice. (F) Hypothetical model of changes in the migration of osteoblastic precursor cells during osteogenic differentiation based on the results of this study. The motility of osteoblastic progenitor cells was enhanced at early stages of osteogenic differentiation. At later stages, differentiated cells showed low motility and high adhesion ability. FA: focal adhesion. SF: stress fiber.

cells on day 1 were more motile than undifferentiated cells and move through the activation of Cdc42 and Rac1, which is critical for mesenchymal mode of migration. The mesenchymal mode of cell migration has been reported to be enhanced by the inhibition of Rho-ROCK-myosin signaling [18–21]. We therefore next determined of the effect of ROCK inhibitor, Y-27632, on ST2 migration.

3.2. A mesenchymal mode of cell migration was promoted by inhibition of ROCK signaling

The experimental protocol is shown in Fig. 2A. To determine if the ST2 cells migrated using a mesenchymal mode of migration, we compared parameters of migration in cells treated with Y-27632 and/or rhBMP-2 for 1 day. ALP activity was enhanced by treatment with Y-27632 as well as by rhBMP-2. Treatment with both rhBMP-2 and Y-27632 resulted in marked high ALP activity (Fig. 2B). Cell shape was also changed by treatment with Y-27632 (Fig. 2C and D) and Y-27632-treated cells showed enhanced cell protrusion compared to rhBMP-2 treated-cells (Supplementary movies 3 and 4). This shape change was accompanied by attenuation of actin stress fibers and focal adhesion formation suggesting enhanced cell migration (Fig. 2E). Treatment with both rhBMP-2 and Y-27632 resulted in morphological changes similar to those observed following Y-27632 treatment alone. Cell migration in the wound healing assay was enhanced by treatment with Y-27632 as well as with rhBMP-2, but an additive effect of Y-27632 was not detected (Fig. 2E). We speculated migration speed of ST-2 was maximized at the BMP-2 treatment with/without Y-27632. By contrast, ALP activity was increased when we used rhBMP-2 with Y-27632. Y-27632 treatment stimulated the production of endogenous murine BMP-4 in ST2 cells (Supplementary data, Fig. S3). We also found that ALP activity reached a plateau even increasing amount of only rhBMP-2 (Supplementary data, Fig. S4). We speculated that Y-27632 induced osteogenic differentiation not only by increased BMP production but also by unknown additional mechanism. Thus, the combined treatment showed additive effect for ALP activity in ST2 cells (Fig. 2B). Cell adhesiveness was decreased by treatment with Y-27632 and with both rhBMP-2 and Y-27632, but not with rhBMP-2 alone (Fig. 2F). Finally, the activity of Cdc42 and Rac1 was not changed following treatment with Y-27632 but it was significantly increased by treatment with rhBMP-2 (Fig. 2G). These data indicated that adhesiveness and cell

morphology during osteogenic induction were different between Y-27632-treated and rhBMP-2-treated cells. These differences may reflect different mechanisms by which these treatments induce a mesenchymal mode of cell migration. Thus, while the rhBMP-2-treated cells did not modulate cells adhesion, these cells showed enhanced cell ruffling as well as cell protrusion compared to controls (Supplementary movies 1–4), which is the characteristic shape of mesenchymal mode migrating cells. In contrast, the Y-27632-treated cells showed decreased cell adhesion and enhanced cell protrusion compared to the rhBMP-2-treated cells. These results suggested that treatment with Y-27632 induced a mesenchymal mode of cell migration by inducing a decrease in cell adhesiveness.

We further examined the role of RhoA signaling in the mesenchymal mode migration of the cells by inhibition of the Rho-ROCK-myosin signal using blebbistatin, a specific myosin ATPase inhibitor. The proliferation of ST2 cells was not changed by treatment with either Y-27632 (10 μM) or blebbistatin (10 μM) (data not shown). Wound healing was accelerated by treatment with either Y-27632 or blebbistatin (Fig. 3A). The phosphorylation level of MLC decreased following treatment with Y-27632, but was not changed by blebbistatin treatment. We also found ALP activity was not increased by treatment with blebbistatin (Supplementary data, Fig. S5). Thus, we speculated that the inhibition of Rho-ROCK-myosin signaling with blebbistatin increased cell migration without osteogenic induction. On other hand, inhibition of ROCK with Y-27632 enhanced both osteogenic induction and cell migration in undifferentiated cells. To further analyze the effects of Y-27632 and blebbistatin on mesenchymal cell migration, we prepared primary mouse mesenchymal cells from bone marrow and expanded these BMDCs in vitro. These cells showed the characteristic spindle-shape of mesenchymal stem cells on the plastic culture dish (Fig. 3B) and could differentiate into osteoblasts or adipocytes under the appropriate culture conditions (Fig. 3C). Treatment with either Y-27632 or blebbistatin increased wound healing of primary BMDCs (Fig. 3D). These results indicated that inhibition of ROCK signaling that is downstream of RhoA, induced increased migration, not only of ST2 cells, but also of BMDCs. In order to evaluate whether Y-27632 promoted migration of undifferentiated or differentiated mesenchymal cells, we examined whether Y-27632 promote migration of differentiated ST2 cells and MC3T3-E1 preosteoblast cell line in vitro. Y-27632 did not promote cell migration of differentiated cells (Fig. 3E and F). Thus, we speculated that the effect of Y-27632 is specific to undifferentiated cells.

3.3. Upregulated migration of progenitor cells increased ectopic bone formation

We lastly investigated the effect of Y-27632 and rhBMP-2 treatment on cell migration and bone formation in vivo. We implanted a rhBMP-2-collagen1 composite into a dorsal subfascial pocket and observed ectopic bone formation. For analysis of the effect of Y-27632, this inhibitor was continuously released from an osmotic pump (Fig. 4A). Using HE staining and in situ hybridization, recruitment of osteopontin positive cells into the rhBMP-2-collagen1 composite was observed, 4 days after implantation in Y-27632-treated mice, but not in control mice (Fig. 4B). We also observed bigger calcified ossicles over a period of 1 week in the Y-27632-treated group compared to the control group, using HE staining of histological sections of the BMP composite (Fig. 4C), macroscopic imaging of ectopic ossicles (Fig. 4D) and radiographic analysis of the ossicles (Fig. 4E). These results suggested that the migration ability of undifferentiated mesenchymal cells was upregulated by in vivo treatment with Y-27632 and resulted in enhanced bone formation.

In summary, we propose a hypothetical model to explain the findings of this study (Fig. 4F). Firstly, when progenitor cells receive an osteogenic differentiation signal such as BMP-2, cell morphology changes over time and cell motility is temporary enhanced via activation of both Cdc42 and Rac1. Subsequently, motility gradually decreases, day by day, following differentiation. At a later stage, the differentiated cells have low motility and high adhesion ability via the low turnover of focal adhesion. Thus, cell migration dramatically changes at each stage of differentiation. We speculated that inhibition of Rho-ROCK-myosin signaling leaded to make a change of balance between Cdc42/Rac1 and RhoA activity, which induced a mesenchymal mode of cell migration. Further study is necessary to investigate the precise factors that control these phenomena. Elucidation of the link between migration and differentiation could provide useful future clinical applications for bone formation, fracture healing, and regenerative medicine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.11.014.

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